Pancreas-derived mesenchymal stromal cells share immune response-modulating and angiogenic potential with bone marrow mesenchymal stromal cells and can be grown to therapeutic scale under GMP conditions

Cytotherapy

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Abstract:	Background Mesenchymal Stromal Cells (MSC) isolated from various tissues are under investigation as cellular therapeutics in a wide range of diseases. It is appreciated that the basic biological functions of MSC vary depending on MSC tissue source, however in-depth comparative analyses between MSC isolated from different tissue sources under good manufacturing practice (GMP) conditions is lacking. Aims and Objectives Human clinical grade, low purity islet (LPI) fractions are generated as a by-product of islet isolation for transplantation. MSC isolates were derived from LPI fractions with the aim to perform a systematic, standardised comparative analysis of these cells to clinically relevant bone marrow-derived MSC (BM MSC). Materials and Methods We derived MSC isolates from LPI fractions and expanded them in platelet-lysate (PL)-supplemented medium, or in commercially available defined xeno-free medium. We compared doubling rate, phenotype, differentiation potential, gene expression, protein production and immunomodulatory capacity of LPI to BM MSC. Results and Conclusion We show that MSC can readily be derived in vitro from the non-transplanted fractions from islet cell processing (LPI MSC). LPI MSC grow stably in serum-free or PL-

	supplemented media and demonstrate in vitro self-renewal as measured by colony forming unit assay (CFU-F). LPI MSC express similar patterns of chemokines and pro-regenerative factors to BM MSC and importantly, are equally able to attract immune cells in vitro and in vivo and suppress T cell proliferation in vitro. Additionally, we show that LPI MSC can be expanded to therapeutically relevant doses at low passage under GMP conditions and therefore represent an alternative source of GMP MSC with functions comparable to BM MSC.

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Cytotherapy Journal

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Re: JCYT-D-00242-R4

Dear Dr Phinney

Thank you for your positive response to our revised manuscript.
We have revised the manuscript as requested, by changing the term in the abstract.

Yours sincerely

John Campbell

Response to reviewers

JCYT-D-00242-R4
Changes in the text are highlighted in red throughout the revised manuscript.

Reviewers' comments:

The authors have addressed reviewer comments satisfactorily. One correction is requested: The reworked abstract states that LPI-MSCs demonstrate "stemness" as measured by colony forming unit assay. I would suggest this be revised to state that LPI-MSCs demonstrate in vitro self-renewal as measured by colony forming unit assay in keeping with ISCT's position paper on assays required to demonstrate stem cell properties (Viswanathan et al., 2019 Cytotherapy).

This has been re-written as requested

Pancreas-derived mesenchymal stromal cells share immune response-modulating and angiogenic potential with bone marrow mesenchymal stromal cells and can be grown to therapeutic scale under GMP conditions

Running Title – Pancreatic Islet MSC for Therapeutic Use

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Key Words: Good Manufacturing Practice; mesenchymal stromal cell; pancreatic islet;

xeno-free; chemokines; T cell suppression

Abstract:

Background

Mesenchymal Stromal Cells (MSC) isolated from various tissues are under investigation as cellular therapeutics in a wide range of diseases. It is appreciated that the basic biological functions of MSC vary depending on MSC tissue source, however in-depth comparative analyses between MSC isolated from different tissue sources under good manufacturing practice (GMP) conditions is lacking.

Aims and Objectives

Human clinical grade, low purity islet (LPI) fractions are generated as a by-product of islet isolation for transplantation. MSC isolates were derived from LPI fractions with the aim to perform a systematic, standardised comparative analysis of these cells to clinically relevant bone marrow-derived MSC (BM MSC).

Materials and Methods

We derived MSC isolates from LPI fractions and expanded them in platelet-lysate (PL)-supplemented medium, or in commercially available defined xeno-free medium. We compared doubling rate, phenotype, differentiation potential, gene expression, protein production and immunomodulatory capacity of LPI to BM MSC.

Results and Conclusion

We show that MSC can readily be derived *in vitro* from the non-transplanted fractions from islet cell processing (LPI MSC). LPI MSC grow stably in serum-free or PL-supplemented media and demonstrate in vitro self-renewal as measured by colony forming unit assay (CFU-F). LPI MSC express similar patterns of chemokines and pro-regenerative factors to BM MSC and importantly, are equally able to attract immune cells *in vitro* and *in vivo* and suppress T cell proliferation *in vitro*. Additionally, we show that LPI MSC can be expanded

to therapeutically relevant doses at low passage under GMP conditions and therefore represent an alternative source of GMP MSC with functions comparable to BM MSC.

Abbreviations:

AD – Adipose

B cell – B Lymphocyte

BM – Bone Marrow

CFU - Colony Forming Unit

DM – Dulbecco's Modified Eagle Medium

DMPL – DMEM plus PL

EMT - Epithelial Mesenchymal Transition

FABP-4 - Fatty Acid Binding Protein-4

GMP - Good Manufacturing Practice

GvHD – Graft versus Host Disease

IDO - Indoleamine, 2-3 Dioxygenase

IEQ – Islet Equivalent

IL-1β - Interleukin-1-beta

IFN-γ - Interferon-gamma

ISCT – International Society for Cellular Therapy

LPI – Low Purity Islet Fraction

MSC - Mesenchymal Stromal Cell

NK cell – Natural Killer Cell

PBMC - Peripheral Blood Mononuclear Cell

PDL-1 - Programmed Death Ligand 1

PGE-2 - Prostaglandin E2

PL – Platelet Lysate

RT – Room Temperature

SM – Stem MACSTM XF Complete medium

SMPL – SM plus PL

SMXF- STEM MACS MSC expansion medium XF

SNBTS - Scottish National Blood Transfusion Service

T cell – T lymphocyte

T-1DM – Type-1 Diabetes Mellitus

TGF-β - Transforming Growth Factor Beta

TNF-α - Tumor Necrosis Factor Alpha

TSG-6 – Tumour Necrosis Factor -Inducible Gene 6

VEGF - Vascular Endothelial Growth Factor

WBC - White Blood Cells

Introduction

Mesenchymal stromal cells (MSC) are multipotent cells found in variable numbers in the majority of tissues. Their immunoregulatory, pro-regenerative and differentiation potential, coupled with their relatively ease of procurement has made them attractive as cellular therapeutics (1). To date, there are over 1300 registered clinical trials (ClinicalTrial.gov) using MSC isolated from a variety of tissues in a wide range of disease and transplant settings. Historically, the majority of the clinical data has been generated from bone marrow (BM)-derived MSC, therefore much of the understanding of MSC function relates to BM-derived cells. While living donor-BM donation is well-established, BM aspirates contain relatively low numbers of MSC that require extensive expansion to reach therapeutic doses (2) and the ease of generating therapeutically relevant doses of functional MSC reduces with increasing donor age (3, 4). BM MSC expanded at large scale show a degree of phenotypic and functional variation over time (5). These passage-dependant functional and phenotypical changes may underlie an observed decline in *in vivo* function, - for example BM MSC are more efficacious when used at lower passage in patients with graft versus host disease (GvHD) (6). As a result, BM MSC may not be ideal for every therapeutic situation and therefore there has been a concerted effort to look for alternative tissue sources, including adipose tissue and umbilical cord (7-9). MSC isolated from different sources are not identical in their biological function, with differences including immune-suppressive ability and angiogenic potential (10-13), this is extremely important when considering MSC therapeutic capacity (13),

When contemplating MSC therapeutic modes of action, it is likely that MSC anti-inflammatory function works in concert with MSC tissue-building capacity. These functions are elaborated in part by chemokines, vital in attracting immune cells such as monocytes (CCL2) and neutrophils (CXCL2) (12); immune modulating factors such as prostaglandin E2

(PGE-2) (14) and indolamine 2,3-dioxygenase (IDO) (15); and a variety angiogenic factors including vascular endothelial growth factor (VEGF) and CXCL8 (13). These properties of MSC are amplified by licensing with various stimulatory factors including interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) and/or interleukin 1-beta (IL-1β). Response to these cytokines can be variable depending on the tissue source (16-18). We recently conducted a comparative analysis of adipose and umbilical cord-derived MSC for differential expression of a suite of chemokines, immune modulating and angiogenic factors (13). Anti-inflammatory and pro-angiogenic phenotypes correlated with positive outcomes in a transplant model (13). These methods are yet to be applied to MSC derived from other tissues.

Pancreatic-derived MSC have been described by a number of groups (18, 19). They can be isolated from the waste product of pancreatic islet transplantation. Islet transplantation is used to treat individuals with Type-1 diabetes mellitus (T-1DM) with unstable glycaemic control (20,21). It is an efficacious based treatment involving enzymatic dissociation of the donor pancreata under GMP conditions to release islets for transplantation (21). Highly pure islets are transplanted into the recipients, leaving a fractionated by-product of digested exocrine tissue and low purity islets - small numbers of islets plus attached exocrine tissue – (LPI). In this study we investigated the potential of using the LPI fraction as a starting material for GMP-compliant manufacture of MSC. We determined that LPI material can be used to manufacture MSC under GMP conditions at scale (LPI MSC). LPI MSC expanded using GMP-compatible reagents were systematically evaluated for their pro-regenerative and inflammatory modulating function in comparison to BM MSC *in vitro* and *in vivo.*

Materials and Methods

Tissues and blood samples

Research protocols and adherence to donation and ethical consent specific to the tissues used in this study were regulated by the Scottish National Blood Transfusion Service (SNBTS) Research Sample Governance Committee.

Non-transplantable LPI were collected from waste fractions of the pancreatic islet transplant process, following processing of donated organs for clinical transplant (20, 21). These tissues were made available for research following informed written consent and their use was governed under SNBTS sample governance reference numbers 12-16 and 15-21. Human volunteer-donor Buffy Coat was used as a source of peripheral blood mononuclear cell (PBMC) for T lymphocyte (T cell) responder assays and chemotaxis assays and were obtained from SNBTS blood processing under sample governance reference number 14-02.

Human Platelet Lysate

Platelet lysate (PL) supplement was produced by repeatedly freezing and thawing date-expired Human Platelet packs (SNBTS) to -80°C for 12 hours and thawing at room temperature (RT). The freeze/thaw cycles were repeated 3 times. Upon final thaw, 10 platelet donation packs were pooled and centrifuged at 350g, before decanting the supernatants as 50ml aliquots and storing at -40°C.

Culture Medium

LPI MSC were derived, maintained and compared in the following culture media; 1. DMPL-DMEM (Thermo Fisher Scientific), supplemented with Heparin (Leo Labs) at a final concentration of 2IU/ml, 1×Non-essential Amino Acids (Thermo Fisher Scientific), 5% human platelet lysate. 2. SM- STEM MACS MSC expansion medium XF (SMXF) (Miltenyi

Biotec Ltd.). 3. SMPL- SMXF supplemented with 5% human PL. Unless stated otherwise, studies show LPI and BM MSC maintained in SMPL.

Tissue Processing and Culture Initiation

Pancreatic Material

Waste LPI fractions were received from the SNBTS islet isolation lab. LPI was washed once in SMPL medium, centrifuged at 300g for 5 minutes and then cultured at 0.006ml/cm^2 at 37°C in 5% CO_2 in SMPL medium. (e.g. 0.45mL LPI fraction in a T75 flask, plus 9.5ml SMPL medium). Explant outgrowth was assessed, and adherent cells were observed migrating from the explanted materials. The medium was carefully exchanged at day 7 and thereafter changed every 3-4 days. Cultures were observed and photographed using an EVOS Cell Imaging System (Thermo Fisher Scientific). Once the cultures had reached 80-90% confluence the cells were recovered with a 10 minute incubation at 37°C with 0.13ml/cm^2 1 × TrypLE Select (Thermo Fisher Scientific). To remove cell debris the material was passed through a 100µm cell strainer (Falcon). The cells were counted using a haemocytometer and designated as "passage 0". These cells were either cryopreserved at 1×10^6 / 2ml cryovial in Cryostor (CS10, Sigma Ltd) or re-cultured at a density of 3000 cells per cm^2 in Corning CellBIND flasks.

Intensification of MSC Manufacturing Density

Five donations of LPI were processed at a higher re-seeding density of 5000 cells per cm^2 in SM +/- PL medium only at passage 1 and passage 2.

Bone Marrow MSC

Existing stocks of BM MSC isolates were used in this study for comparison. These cell populations had been previously generated up to passage 3 using standard methods (1).

Medium change/passage

All media were changed twice per week. On reaching 80-90% confluence, cultures were collected as described above, the cell count and yield per flask determined and re-seeded at a density of 3000 cells per cm^2 in Corning CellBIND flasks. Cells were expanded continually in culture until at least 3 passages of complete cycles of growth to confluence had been achieved after passage 0. Unless stated otherwise, LPI and BM MSC were used at P3 throughout this study.

Tri-lineage differentiation

MSC were assessed for their differentiation capacity using the Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems). This kit contains all necessary differentiation supplements and the primary and secondary antibodies required for detection. The mature phenotype of adipocytes, chondrocytes and osteocytes were defined by the binding of antibodies against fatty acid binding protein-4 (FABP-4), aggrecan and osteocalcin respectively. Primary antibodies were detected using secondary antibodies specific to the primary antibody (Northern Lights 577-conjugated anti-goat – FABP-4, Northern Lights 557-conjugated donkey anti-mouse – osteocalcin and Northern Lights 557-conjugated Donkey Anti-goat –aggrecan). Samples were imaged with a Zeiss epi-fluorescent microscope and prepared using Zeiss software.

Flow Cytometry

Cells were dissociated into a single cell suspension and washed twice in buffer comprising PBS /2mM EDTA /0.1% human serum albumin (flow buffer). For MSC phenotyping, cells were stained using antibodies at various concentrations (detailed in table S1) in a total volume of 100ul for 15 minutes at 4°C. Cells were washed 1× in flow buffer and resuspended in 200µl of flow buffer for analysis. Voltages were set using fluorescence minus one controls. A

minimum of 10,000 events were collected. Flow cytometry analysis was performed using BD LSRFortessa (BD Biosciences) or MACS Quant and analysed with Flow Jo software (Treestar-Ashland, Oregon, USA).

T cell suppression assay

T cell suppression assays were carried out as previously described (13) using ratios of MSC:PBMC ranging from 1:2 to 1:16.

Chemotaxis assay

Whole white blood cells (WBC) were isolated from fresh buffy coat as detailed in supplemental material 1. MSC were seeded at 3000 cells/cm^2 in DMPL and grown as a monolayer in 24 well plates (Corning). Once 80% confluence was reached, MSC were either left unlicensed, or licensed. Licensing of MSC was carried out by incubation of 80% confluent cultures in complete medium supplemented with 10ng/ml each, of IFN-γ, IL-1β and TNF-α (R&D systems). After 24 hours, all wells were washed twice with PBS to remove cytokine, then 600µl of fresh DMPL was added to all wells and left for a further 24 hours. 5µm-pore inserts (Fisher Scientific) were placed into the wells on top of the MSC and 5.5×10^5 WBCs in 100µl of DMPL was placed into the insert. The transwell plate was incubated at 37ºC for 3 hours before the inserts were carefully removed and discarded. Migrated cells were harvested by collecting the supernatant and washing wells thoroughly with PBS, ensuring the collection of loosely adherent cells. Cells were washed and prepared for flow cytometry using Antibodies detailed in table S1. CountBright beads (50µl), used as per manufacturer's instructions (Life Technologies) were added for cell counting.

Murine Air Pouch Model

A previously established air pouch model was used to assess *in vivo* leukocyte migration induced by transplantation of human MSC (35). (Details of husbandry, licenses and techniques in supplemental methods 2). 1×10^6 unlicensed or licensed LPI or BM MSCs in 1 ml of sterile PBS or sterile PBS alone (control animals) was injected into the air pouch 24 hours after the last injection of air. Mice were sacrificed and cells collected as previously described (summarized in S2) after 24 hours. Each sample was split into two and stained for 2 separate flow cytometry panels, one to identify mouse innate immune cells; and one to identify mouse adaptive immune cells; as detailed in table S1.

Gene Expression

LPI and BM MSC were plated at a density of $1\times10^5/cm^2$ in DMPL. Once MSC reached 80% confluence, MSC were licensed as previously described, or left as unlicensed controls. Cells were incubated for a further 24 hours, and then were harvested as above. Supernatants were frozen at -80°C for Luminex analysis of protein expression (see below).

Expression of chemokine, cytokine, chemokine receptor and cytokine receptor genes were assessed using quantitative PCR and RT^2 Profiler™ PCR Arrays Human Chemokines & receptors (Qiagen) as previously described (13).

Protein Secretion

The 24 hour conditioned media from identical samples used for transcript analysis were collected and analysed using a Luminex 100 analyser (Bio rad) and premixed magnetic multi-analyte kits (R&D systems cat No. LXS-AHM-2) in accordance to the manufacturer's instructions. All reagents and standards were included in the kit and prepared as outlined in the guidelines. Briefly, samples were diluted 2 fold with calibrator diluent (75µl in 75µl). 10 µl of the pre-coated microparticle cocktail was added to each well of the 96 well microplates,

followed by either 50µl sample or 50µl standard, sealed and placed on an orbital shaker (0.12mm orbit at 800 ± 50rpm) for 2 hours at room temperature (RT). The plates were washed twice with 100 µl/well wash buffer and then incubated with 50 µl/well anti-biotin detector antibody for 1 hour at RT on the shaker (0.12mm orbit at 800 ± 50rpm). The plates were washed as before and 50 µl/well of streptavidin-phycoerythrin was added and incubated for 30 minutes at RT. Microparticles were re-suspended in 100 µl/well of wash buffer and immediately read on the Bio-Rad analyser. Each microparticle bead region was designated and doublets excluded as stated on the certificate of analysis.

Statistics

Graphs and statistical analysis were generated with GraphPad Prism 6. Unpaired T tests were used to compare LPI vs. BM material and Paired T test's utilised when assessing statistical differences within one tissue source. Significant differences are marked on individual figures and represented as *$p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Results

Establishment of human LPI cultures in GMP -compliant medium

In culture, LPI material initially presented as plastic-adherent islets, identified through positive dithizone staining (Fig. 1.a.ii), and islands of dithizone negative exocrine tissue (Fig. 1.a.iii). Adherent, cobble-stone shaped cells grew out from the islets and exocrine tissue as a monolayer, with longer spindle-shaped cells at the outer edges of the monolayer (Fig. 1.a). Flow cytometric analysis of freshly isolated tissue (day 0), showed that the majority of cells were EPCAM +ve epithelial cells, and no CD90 and CD105+ve cells were detected (Fig. 1.b.i). As cultures matured, EPCAM and MSC marker expression was mutually exclusive and the prevalence of CD105, CD90 and CD73 cells went from <1% to >90%, and EPCAM expressing cells went from >50% to <0.3% over a period of 16 days (Fig. 1.b.i-iv). At P0, the majority of cells were positive for MSC markers, where 95% of cells were vimentin +ve and <1% were EPCAM +ve (Fig. 1.b.iv).

LPI derived cells could be reliably established in GMP-compliant media; DMPL, SM and SMPL, where doubling rate was consistent across all three passages (P1-P3) (Fig. 2.a). LPI MSC grown in SMPL however, returned significantly higher CFU-F at P2 and P3, compared to DMPL and SM (Fig 2.b). There were no differences in CFU-F between LPI MSC and BM MSC grown in in SMPL at any of the three passages assessed (Fig 2.c). LPI cells established in SMPL displayed a characteristic MSC-like phenotype through plastic adherence and spindle-shaped morphology, akin to that of BM derived MSC grown in SMPL (Fig. 3.a). LPI cultures also expressed all the relevant MSC markers including positive expression of CD73, CD90 and CD105 and no expression of CD45, CD19, CD11b, CD34, CD14 and CD31. This MSC surface marker expression was maintained and EPCAM expression was consistently lacking through all passages (P3-P5 illustrated fig.3.b.). LPI derived-cells expression levels of all the aforementioned markers were similar to BM MSC at P3 (Fig. 3.b). Finally, to

confirm that LPI-derived cells were MSC, LPI cultures were differentiated into the three classical lineages; bone, cartilage and adipose. Positive expression of FAB-4 (Fig.3.c.i), osteocalcin (Fig.3.c.ii) and aggrecan (Fig.3.c.iii) confirmed successful differentiation into all three lineages, thus LPI cultures were considered MSC-like cells and are referred to as LPI MSC throughout the text.

LPI MSC and BM MSC express a range of immune modulatory and pro-regenerative factors

To understand whether LPI derived MSC displayed therapeutically desirable regenerative and immunomodulatory potential, they were assayed for their transcriptional and protein expression of immune response modulating and pro-regenerative factors with and without licensing, and compared to BM MSC at the same passage (Fig. 4.a and b). Unlicensed LPI and BM MSC expressed similar transcriptional patterns of chemoattractant/inflammatory modulating molecules, with the exception of CX3CL1 (fractalkine) and IL-16, which were transcribed at marginally higher levels in the LPI MSC. Upon licensing, both MSC types uniformly upregulated the inflammatory regulators tumor necrosis factor-inducible gene 6 (TSG-6) and IDO. Patterns in the transcript expression of the pro-angiogenic CXC chemokines were almost identical between LPI and BM MSC, where CXCL8 was the most highly transcribed pro-angiogenic gene in LPI and BM derived MSC, with or without licensing (Fig. 4.a.). Various monocyte (CCL2), macrophage (CCL4), dendritic cell (CCL20), and neutrophil chemoattractants (CXCL's 1, 2, 3, 5, 6 & 8) were all upregulated upon LPI and BM MSC licensing (Fig. 4.a.). Conditioned media from the same cells used in transcriptional analyses were tested for a selection of pro-angiogenic and chemotactic factors. Secreted proteins from licensed LPI and BM MSC closely tracked the transcriptional observations, with high amounts of CCL2, CCL20 and CXCL8 detected in unlicensed MSC

supernatants which were markedly upregulated upon licensing. VEGF, a pro-angiogenic factor was also produced in moderate amounts by resting and licensed MSC from either source (Fig. 4.b.).

Immune cell attraction profiles of LPI and BM MSC are comparable both *in vitro* and *in vivo*

Chemotaxis assays were utilized to assess whether LPI and BM MSC were able to induce migration of immune cells. Unlicensed LPI MSC differed markedly from BM MSC in attracting significantly more CD45 +ve cells, specifically neutrophils and monocytes. Upon licensing, both LPI and BM MSC attracted neutrophils and monocytes, with little to no attraction of B lymphocytes (B cells), T cells, natural killer (NK) cells and eosinophils (Fig. 5.a.). LPI MSC attracted proportionately more monocytes than BM MSC but this did not reach statistical significance. To establish if observed *in vitro* behaviors of LPI and BM MSC persisted *in vivo*, this analysis was extended to a murine air pouch model. In contrast to *in vitro* data, unlicensed LPI and BM MSC attracted similar total numbers of all immune cells, with no significant differences detected. As observed *in vitro*, licensing LPI and BM MSC resulted in a marked upregulation in the ability of MSC to induce migration of all immune cells. No significant differences in the total number of immune cells migrating towards licensed LPI or BM MSC were observed, with the exception of licensed BM MSC attracting significantly more NK cells than licensed LPI MSC. Notably, similar to *in vitro* migration data, neutrophils and monocytes made up the majority of migrating immune cells towards licensed or unlicensed LPI and BM MSC, however, the migration of moderate numbers of B cells, NK cells and eosinophils were also observed *in vivo* (Fig. 5.b).

LPI MSC are potent suppressors of T cell proliferation

Next, suppression of T cell proliferation by LPI was assessed and compared to BM MSC. We first investigated the capacity of MSC to suppress T cell proliferation without licensing (Example analysis Fig 6.a). Either LPI or BM MSC strongly suppressed T cell proliferation at ratios of 1MSC:2 PBMC, and the effect titrated with reducing numbers of MSC (Fig.6.b). Suppression of proliferation by LPI MSC was significantly higher at 1:8 than with BM MSC. Given that strong suppressive effects were seen with unlicensed MSC, we further investigated the role that licensing plays in MSC-suppression of T cell proliferation. Overall, licensing MSC had no beneficial or detrimental effect on T cell suppression mediated by MSC compared to unlicensed MSC, and no significant differences were observed between LPI and BM MSC at any ratio tested (Fig.6.c).

GMP-compliant method and density intensification - scale up for manufacturing

To scale up for manufacturing, a suitable volume of LPI tissue and subsequent re-seeding densities of LPI MSC had to be determined. Initially, LPI tissue was tested at a density of 0.006ml/cm^2 or 0.03ml/cm^2 (1ml or 5ml total LPI fraction per T175 flask in a total volume of 35ml). Cells were more readily established using the lower seeding density of 0.006ml/cm^2. (data not shown). Therefore, to model a complete manufacturing process, 1 ml of LPI tissue was seeded into a T175 (0.006ml/cm^2), and thereafter re-seeded at 5000 cells/cm^2. The median cell yield at P0 was 13×10^6, with a range of cell yield between 72×10^6 and 4.7×10^6 (Fig. 7.a). MSCs grown as described above reached median yields of 5200×10^6 by P2 (Fig. 7.a). As waste tissue from a successful islet isolation ranges from 6mls to 22mls (Fig. 7.b), theoretical yields of LPI MSC at P2 could range from 37000×10^6 (6mls) to 116000×10^6 (22mls) (Fig. 7.c). Cells manufactured in this way maintained the CD45-ve HLA-DR-ve, CD73+, CD90+ CD105+ phenotype (Fig. 7.d).

Discussion

In this study we have shown that LPI MSC culture can be easily initiated, and the cells can be expanded to therapeutic scale at low passage. We have extensively compared phenotype and function of LPI-derived MSC to BM MSC and show that LPI MSC share therapeutically relevant characteristics with BM MSC.

Derivation of LPI-MSC

MSC-like cells isolated from pancreatic tissue have been described by several groups (18, 19), we sought to expand on this work to produce GMP-grade MSC cell populations, rather than to re-programme these cells e.g. into beta-like cells. *In vitro* expanded pancreatic MSC populations may arise from small numbers of resident MSC, or as a result of epithelial to mesenchymal transition (EMT) and has been explored elsewhere (22-25). The phenotypic changes of LPI isolates we have described in this study point to EMT as the principal mechanism underlying the derivation of LPI MSC cultures. We show that freshly isolated LPI tissue lacks cells expressing the mesenchymal markers CD105 and CD90, but is rich in EPCAM +ve cells. Over the 16 day *in vitro* expansion period to reach P0, EPCAM +ve cells gradually become positive for CD90 and CD105 followed by a reduction in EPCAM staining cells to <1% of the total population, which was maintained throughout subsequent passages. At P0 over 91% of cells express and the definitive mesenchymal marker vimentin. From P1 onwards, the MSC express CD105, CD90 and CD73. It is therefore most likely the LPI-MSC manufacturing processes described in this study produces isolates of culture-induced MSC-like cells as a result of *in vitro* EMT, although involvement of small populations of precursors cannot be entirely ruled out.

Function and Phenotype

The ISCT criteria for definition of MSC (29) has underpinned all recent MSC research and the LPI MSC generated in this study met all of these criteria. Phenotype alone does not reveal whether MSC from different sources, or cultured using different methods have equal therapeutic capacity. We have recently reported that umbilical cord derived MSC when co-transplanted with islets into diabetic mice have a greater benefit on glycaemic control versus adipose derived MSC (cultured identically) and that these MSC isolates differed widely in expression of genes important in immune-response and angiogenesis (13). Here, we took a similar standardised approach in our analysis to systematically compare LPI MSC to BM MSC to ensure that they share clinically applicable characteristics beyond basic phenotyping. Transcriptional analysis of more than 30 genes highlighted that LPI MSC express similar immune-modulatory, pro-angiogenic and chemotactic factors to BM MSC. BM and LPI MSC responded to licensing with an upregulation in a number of genes including the immune modulators TSG-6 (27) and IDO (28), the proangiogenic and neutrophil chemoattractants CXCL2 and CXCL8 (29, 30), and the monocyte chemoattractant (also pro-angiogenic) CCL2 (31).

We have shown that these transcribed genes translate into *in vitro* and *in vivo* activity of the MSC with similar patterns in protein secretion between LPI and BM MSC. *In vitro*, strong chemoattraction of myeloid cells was a function of licensed LPI and BM MSC. *In vivo*, unlicensed BM and LPI MSC both induced infiltration of neutrophils and monocytes into the injection site. This function was greatly amplified when MSC were licensed before injection. Recruitment of inflammatory cells may be an unexpected function of cells with proven anti-inflammatory properties, however, recruitment of circulating monocytes has been shown to be critical for microvascular growth (32). Moreover, neutrophil recruitment is necessary for blood vessel formation in an *in vivo* angiogenesis model (33). The chemoattraction of immune cells towards MSC likely serves more than one purpose, extending beyond

angiogenesis to immunomodulation of these attracted immune cells, presumably altering the inflammatory environment. Here we show that BM and LPI MSC not only attract similar types and numbers of immune cells, they also express similar patterns of the immunomodulatory genes TSG-6, IDO and TGF-β whilst exerting similar capacity to suppress T cell proliferation. The ability of MSC to attract immune cells and subsequently immunosuppress them is an important therapeutic mode of action of MSC which has been demonstrated in a model of GvHD (34). Treatment of GvHD is an area where LPI MSC may offer an advantage over BM MSC, as LPI MSC supressed T cell proliferation at lower T cell:MSC ratios, and attracted more myeloid cells in an unlicensed state. LPI MSC could also potentially be used in islet transplant recipients: MSC have been shown to enhance engraftment and function in experimental models (13). Patients routinely receive two islet grafts (20), hence MSC could be generated from the LPI of the first transplant and used subsequently as an MHC-matched accessory cell to support the second graft, with potentially reduced immune sensitization compared to third-party MSC.

Manufacturing of LPI MSC

Extending the applications of a donated pancreas beyond high purity islet transplantation to involve the manufacture of MSC from the LPI fraction would extend clinical application of the donated organ to potentially treat many patients. Recent registry data indicates >2600 donated pancreata for islet processing (36) (North America, Europe and Australia) over a ten year period to 2015. LPI fractions are therefore routinely available from transplant centres and should be readily available for distribution to manufacturing centres. LPI-MSC grow rapidly, in xeno-free medium – a single T175 flask initially seeded with 1ml of LPI can generate 5.2×10^9 cells after two *in vitro* passages, with theoretical yields from a single LPI fraction of 31.7×10^9 – 116×10^9. This would be sufficient MSC to manufacture 356-1303 doses (based on 1×10^6 per kg with a patient weighing 89kg). This compares favourably to

projected manufacturing yields for BM MSC under GMP conditions using a whole BM donation (20-37ml) and standard culture vessels (2,5): $6.6x10^9$ at P2 and up to $6.3x10^9$ at P1 respectively. Manufacture of MSC, irrespective of source, at this scale would become impractical using standard culture vessels and would benefit from the use of bioreactors – not necessarily to increase yield (this is not guaranteed e.g. ref 37.), but for ease of handling and speed of processing. Manufacturing to very large scale may well not be required in a single centre - a single LPI fraction from a donated pancreas could therefore support manufacturing in a number of different centres from a single donation. Samples from a single isolation, in simple storage medium, are routinely distributed fresh from the SNBTS islet isolation lab around the UK to multiple centres, subject to appropriate consent.

In summary, we have demonstrated that MSC, derived from low purity islets fractions (LPI), exhibit many of the ideal immune-responsive and pro-regenerative functions of MSC *in vitro* and *in vivo.* These cells are readily expanded from donated clinical-grade material that is currently an unused by-product of islet isolation, and we have demonstrated their successful manufacture using fully GMP-compliant materials to therapeutic dose at low passage. Further studies will be required to determine the therapeutic potency of this novel MSC population.

Figure Legends.

Fig.1. GMP MSC culture derivation from waste LPI fractions of the islet isolation process

a. Phase contrast micrographs of cellular outgrowth from islands of tissue isolated from LPI fractions. 14 days in culture results in characteristic cobblestone-shaped cells emerging from dithizone stained islets (ii) and exocrine tissue (iii), forming spindle-shaped MSC-like cells at edges. Scale bar represents 0.2mm.
b. Three parameter flow cytometry analysis of MSC and epithelial markers in LPI fraction over time. Flow cytometry plots show an increase in cells expressing CD90, CD105 and CD73 and a decrease in EPCAM expressing cells over time. An overlay of EPCAM (left column) and CD90 (right columns) show that MSC and epithelial markers are mutually exclusive (i-iii). At P1, LPI isolates lack EPCAM expression and express CD90, CD105, CD73 and vimentin.

Fig. 2. LPI MSC growth characteristics in GMP compliant media and comparison to BM MSC

a. Mean population doubling time for LPI-derived MSC lines in 3 different GMP-compliant media. Mean +/- SD n=7. DMPL – DMEM 5% Platelet lysate, SM – StemMACS, SMPL – StemMACS 5% Platelet lysate.
b. Colony forming unit scores for LPI-derived MSC grown in DMPL, SM and SMPL across 3 passages (p1-p3) with numbers of colonies obtained from plating 10 cells/cm^2 in CFU-F (n=5).
c. Colony forming unit scores for LPI-derived MSC vs BM MSC (n=9), both grown in SMPL across 3 passages. Data presented as mean ± SD and significance marked where applicable, $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Fig. 3. Morphology, Surface marker expression and differentiation capability of LPI MSC

a. LPI isolate at P1 showing plastic-adherent cells with spindle-like MSC morphology (i), similar to that of BM-derived MSC at P2 (ii). SM + PL medium. Scale bar represents 400μm.
b. Representative phenotypes of LPI MSC at P3-P5 (top panels) compared to BM MSC at P3 (bottom panels). LPI MSC homogeneously express CD90, CD105 and CD73, showing similar MFI to BM derived MSC. >99% of LPI MSC lack expression of CD45, CD11b, CD31, CD34, CD19 and CD14. Lack of EPCAM expression by LPI MSC is maintained through passage.
c. Fluorescence micrographs of Tri-lineage differentiation of LPI-derived MSC into Adipose, Bone and Chondrocyte lineages (FAB-4, Osteocalcin and Aggrecan respectively). Matched isotype controls shown in bottom left inserts. Scale bar detailed in each picture.

Fig. 4. Gene and Protein expression of chemoattractant, pro-inflammatory and anti-inflammatory genes in LPI and BM MSC

a. Gene expression by resting and licensed MSCs. The expression of chemoattractant, and proangiogenic genes by resting (-) and licensed (+) LPI and BM MSC (P3) were measured by RT^2 Profiler™ PCR Arrays. The expression of anti-inflammatory genes was measured using qRT PCR as indicated. In each case, the mean $2^{(-\Delta CT)}$ is plotted and heatmaps were generated using Heatmapper software (http://www2.heatmapper.ca/). Each group of genes was analysed separately. n>3<6 donors.
b. Mean levels of chemoattractant and proangiogenic proteins detected by Luminex assay in 24hr supernatants harvested from the cultures detailed above. Data represents total concentration of protein minus background levels of each protein found in medium. Analysed by Heatmapper software as above. n>3<6 donors.

Fig. 5. *In vitro* and *In vivo* immune cell attraction of LPI and BM MSC

a. *In vitro* chemoattraction of peripheral WBC. (i). Representative composition of WBC added to transwell insert at start of chemotaxis experiments. (ii). Migration of WBC to LPI or BM MSC in the unlicensed (-) or licensed (+) state. Data represents the total number of migrated cells minus the total number of background migrated cells and presented as mean ± SEM.
b. *In vivo* airpouch model showing the total numbers of migrated immune cells into the airpouch containing licensed or unlicensed LPI or BM MSC. Data is presented as the total number of each migrated immune cell minus the total numbers in PBS injected control mice. Stacked bars represent mean ± SEM, n= 2 for each MSC donor and 5 mice per group.

Fig.6. Inhibition of T cell proliferation and requirement for IFN-γ licensing

a. Representative dye dilution results measuring inhibition of T cell proliferation by LPI or BM MSC. Cells were grown in SMPL and assayed at P3 and cultured with Ef670-stained PBMC. Ratios are PBMC:MSC at outset of culture.
b. Comparative Inhibition of T cell proliferation by unlicensed LPI or BM MSC. Both MSC types inhibit proliferation and the effects titrate with reducing MSC numbers. Mean of 3 different LPI and 4 different BM lines grown in SMPL, ratios are PBMC:MSC.
c. Comparative Inhibition of T cell proliferation by licensed LPI or BM MSC. Mean of 3 different LPI and 4 different BM lines grown in SMPL, ratios are PBMC:MSC.

Fig.7. Intensification of manufacturing with GMP reagents

a. Total cell yields from 1ml of LPI tissue from P0 to P1 in GMP compliant medium.
b. Total mls of transplanted tissue vs waste tissue in the GMP islet isolation process.

c. Predicted cell yields from the lowest volume of waste tissue (6ml) over 2 passages and the highest volume of waste tissue (22mls) over 2 passages.
d. Flow cytometric analysis of LPI MSC grown using the intensified method demonstrating negativity for hematopoietic markers, and homogeneous expression of CD73, CD90 and CD105. SMPL medium passage 3.

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Supplementary Methods

S.1. WBC preparation.

5mls of donor buffy coat was transferred into a 15ml centrifuge tube. Blood was spun at 300g for 40 minutes (0 break, 0 acceleration). The plasma layer was discarded and the remaining cells were washed with 1x red blood cell lysis solution (Miltenyi Biotec) for 7 minutes, after which cells were spun at 300g for 20 minutes (9 break, 9 acceleration). Supernatant was discarded and remaining cells were washed in 1xPBS. 5ml of buffy coat typically yielded 1.5-2.5x10^8 WBCs.

S.2. Air Pouch Model.

For the air pouch, all animals were housed within the Biological Central Research Facility (University of Glasgow). All experiments received ethical approval and were performed under the auspices of a UK Home Office License. Project licence number: 70/ 8377, Procedure number: 10. All operators held appropriate personal home office licenses. 6 – week old C57BL/6 female mice were obtained from Charles River Europe and before any procedure was carried out, mice were given 7 days within the Biological Central Research Facility for adjustment and settling. After experimental procedures, at the age of 8 – week, mice were euthanised using a recognised Schedule 1 technique (CO_2 followed by femoral artery exsanguination).

Air pouches were generated as previously described (35). Briefly 3ml of sterile air was injected subcutaneously into the intracapsular area of the mouse to create an air pouch. After 3 days, a top-up of 3 ml sterile air was injected into the air pouch. A third top up of 1ml sterile air was injected 2 days later and experimental material was injected 24 hours after the last air injection. Cells or PBS controls were left in the air pouch for 24 hours before mice were sacrificed. Immediately after sacrifice, 3ml of flow buffer was injected into the air

pouches of the mice and mice were gently shaken to allow the flow buffer to mix throughout the air pouch to ensure an optimal retrieval of immune cells.

Table S1.

Monoclonal Antibodies for flow cytometry

Study	Target	Fluorophore	Concentration	Manufacturer
MSC Phenotyping (Anti-human)	CD45	BV421	1:100	Biolegend
	CD19	BV421	1:100	
	CD31	BV421	1:100	
	CD45	BV421	1:100	
	CD11b	BV421	1:100	
	CD14	BV421	1:100	
	EPCAM	BV650	1:100	
	CD73	Pe/Cy7	1:100	
	CD90	APC	1:100	Miltenyi
	CD105	FITC	1:50	
	Vimentin	PE	1:50	
	Viability	Draq 7	1:200	Biostatus
	Fixable viability dye	eFluor™780	1:200	eBioscience
Chemotaxis assay (Anti-human)	Siglec-8	PE	1:100	Miltenyi
	CD4	FITC	1:100	
	CD8	FITC	1:100	
	CD14	VioBlue	1:100	
	CD19	PeVio770	1:100	
	CD66b	APC	1:100	
	CD1C	SA-605	1:100	ThermoFisher
		Biotin	1:50	Biolegend
	CD16	Percp/Cy5.5	1:100	
	CD56	APCCy7	1:100	
	HLA-DR	AF700	1:100	
	Fixable viability dye	eFluor506	1:200	eBioscience
Air Pouch Model Innate immune cells (Anti-Mouse)	CD45	BV421	1:100	Biolegend
	Siglec-F	FITC	1:100	
	F4/80	PE	1:100	
	CD11c	PerCP/Cy5.5	1:100	
	CD11b	Pe/Cy7	1:100	
	Ly6g	APC	1:100	
	Ly6c	APC/Cy7	1:100	
	Viability	eFluor 506	1:100	Biolegend
Air Pouch Model Adaptive immune cells (Anti-Mouse)	CD45	BV421	1:100	
	CD8α	PE	1:100	
	CD4	Pe/Cy7	1:100	
	NK1.1	APC	1:100	
	B220	ApC/Cy7	1:100	
	Anti-human CD105	PerCP Cy5.5	1:100	
	Viability	eFluor 506	1:100	

Figure 1

Fig.1.a

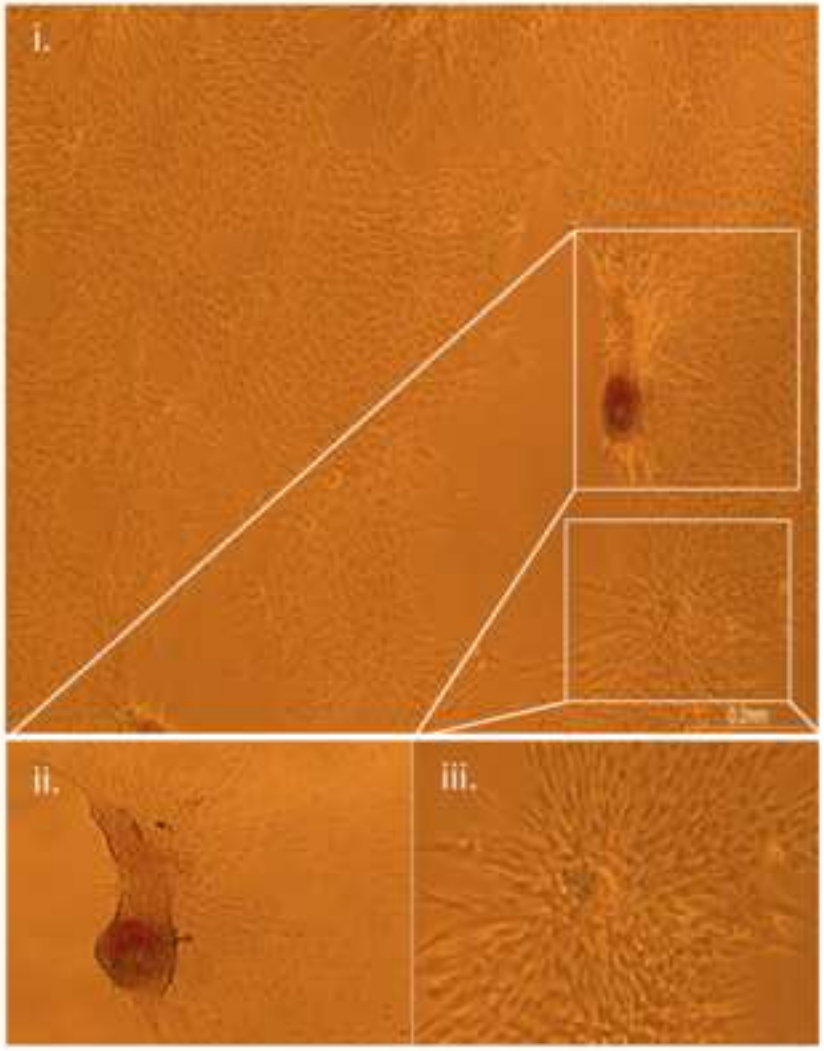


Fig.1.b

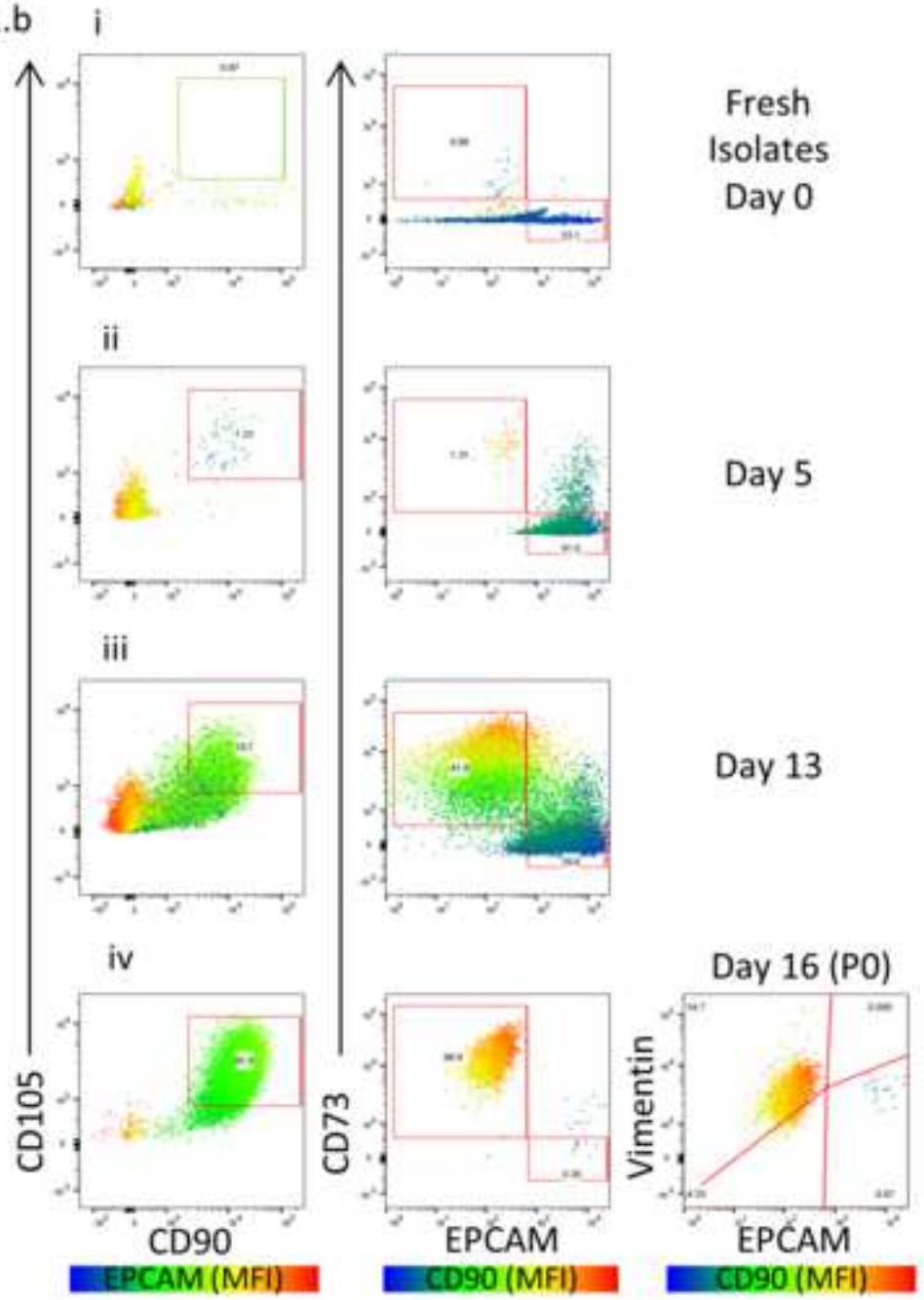
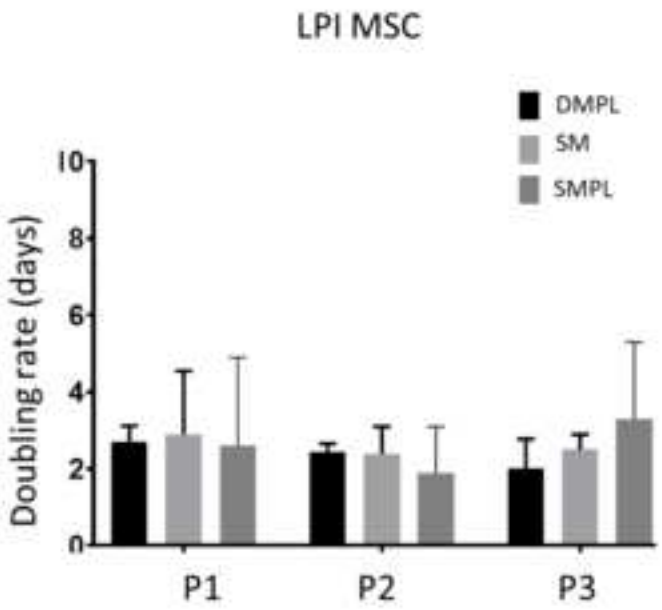


Figure 2

Fig.2.a



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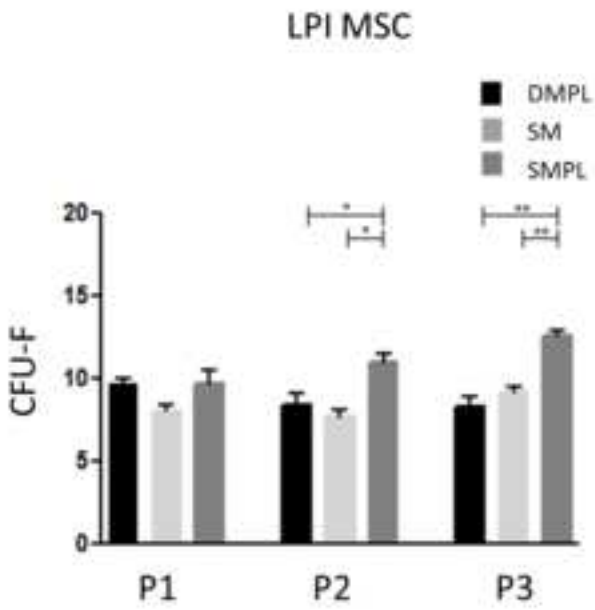


Fig.2.c

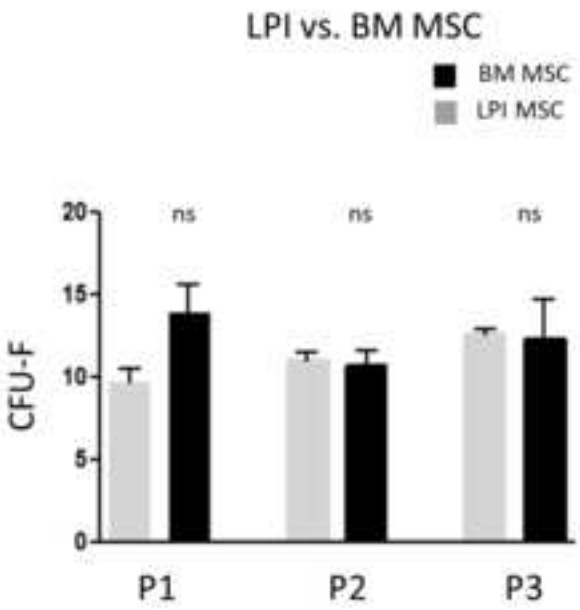


Figure 3

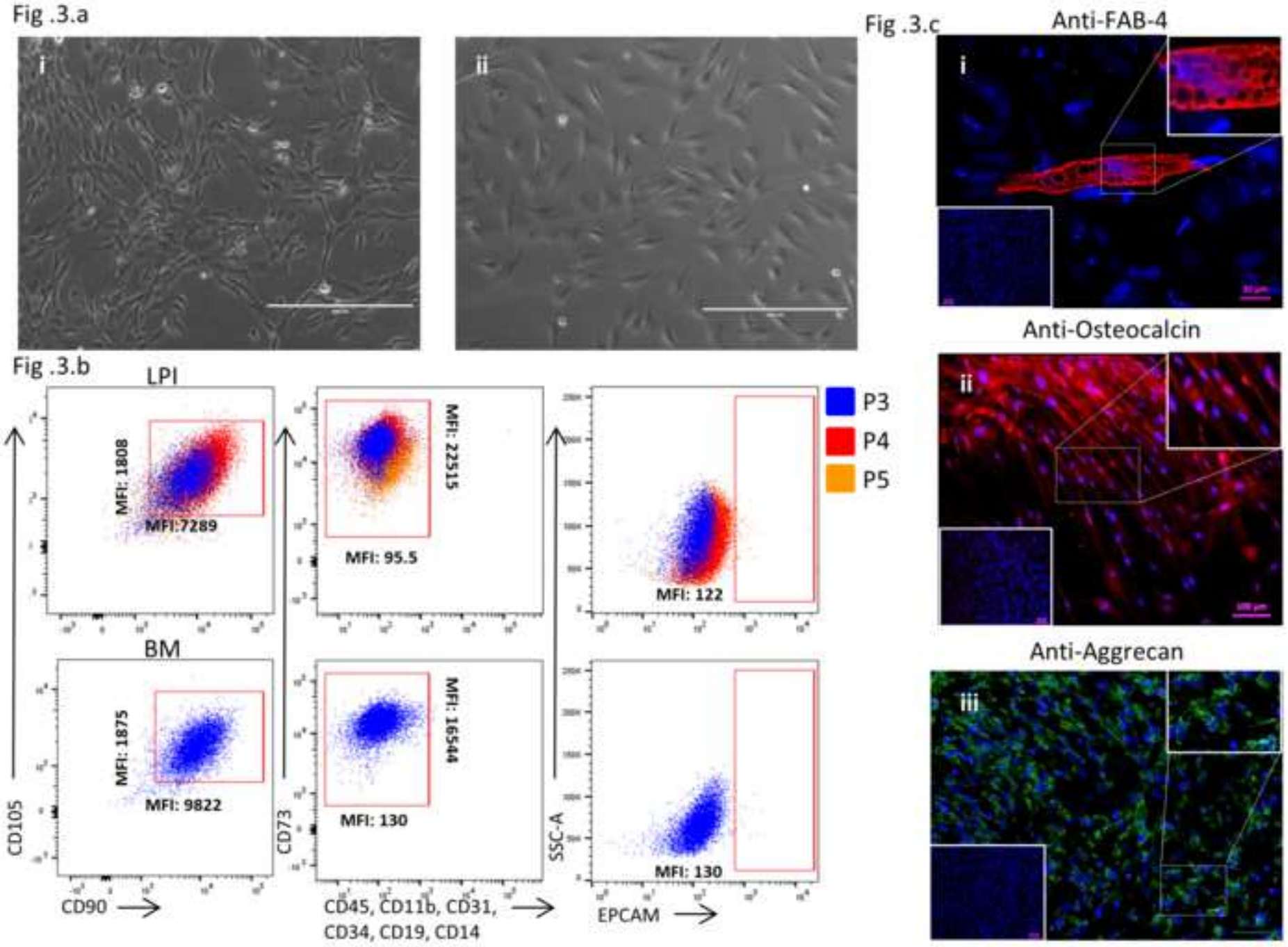


Figure 4

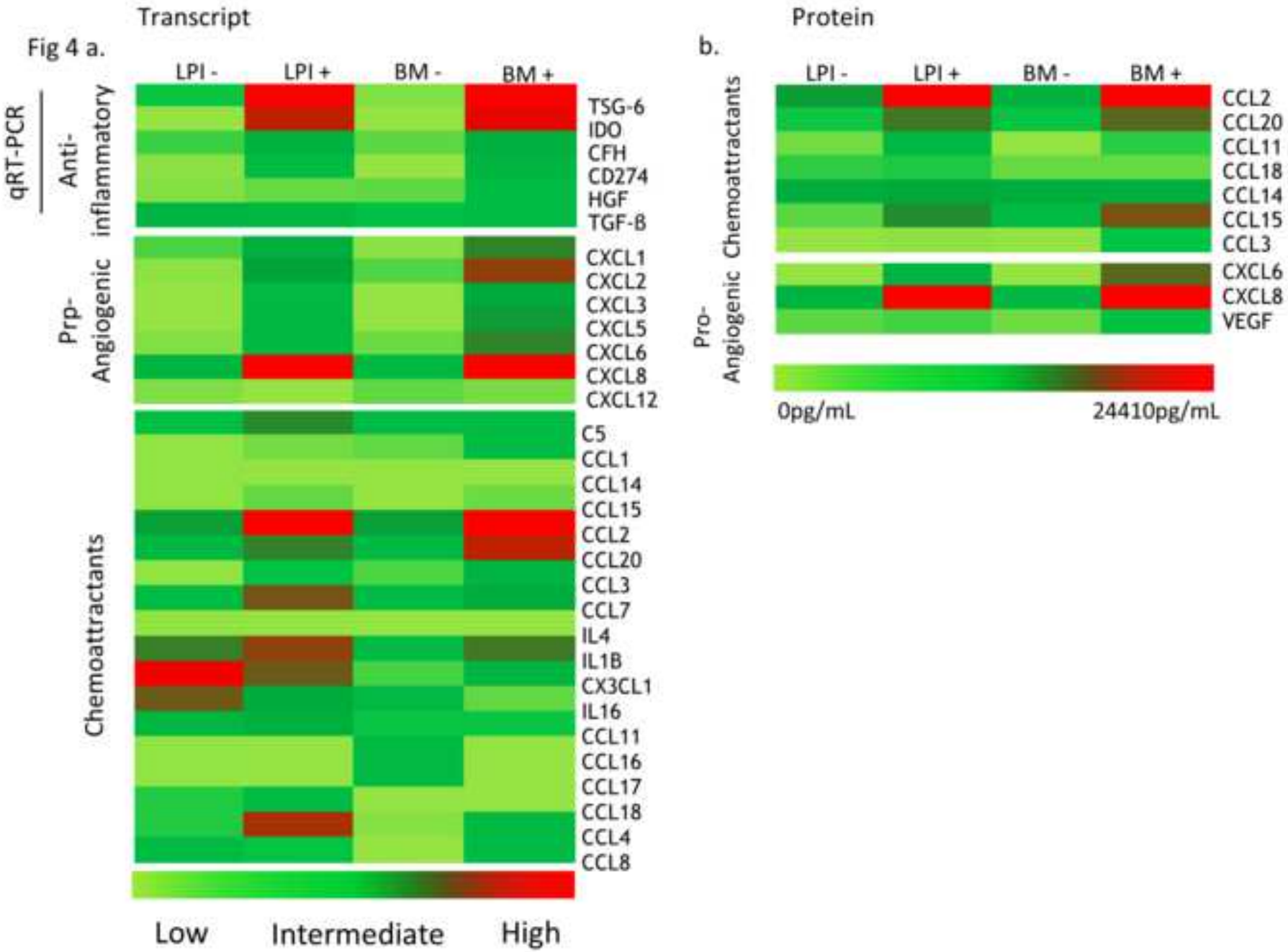
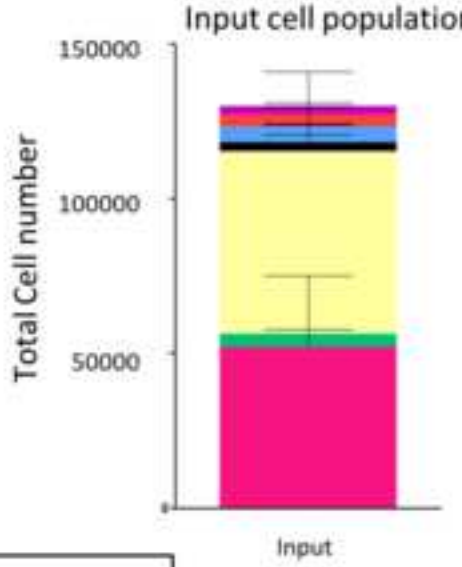


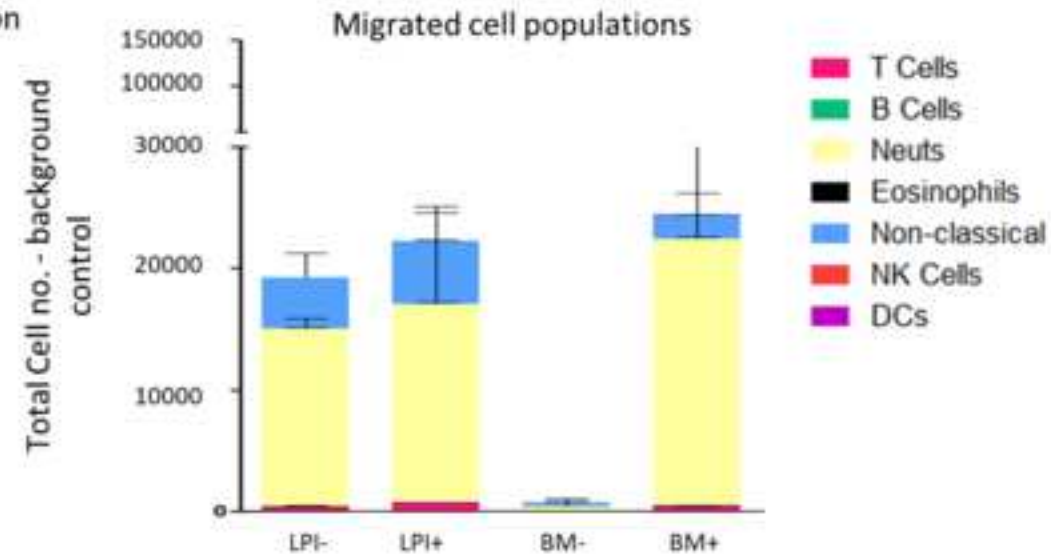
Figure 5

In vitro chemotaxis assay

Fig 5.a..i



ii.



In vivo airpouch model

Fig 5.b

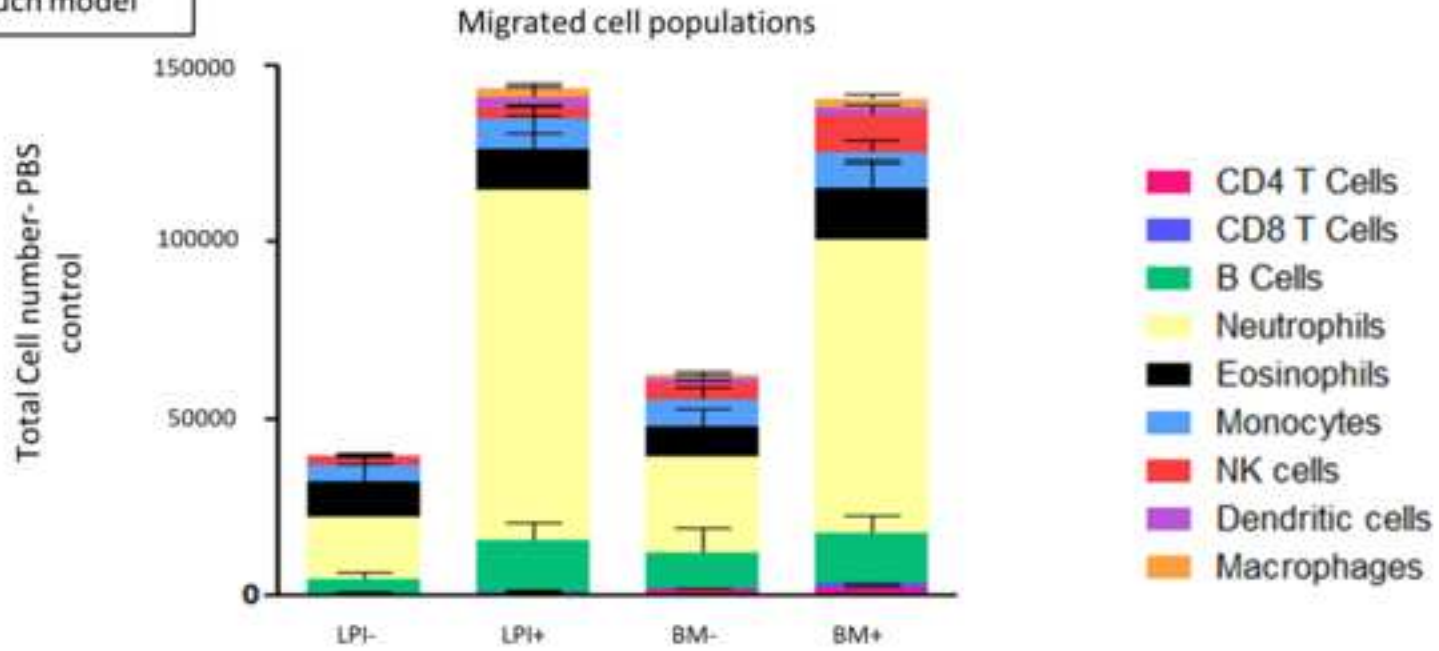


Figure 6

Fig.6.a

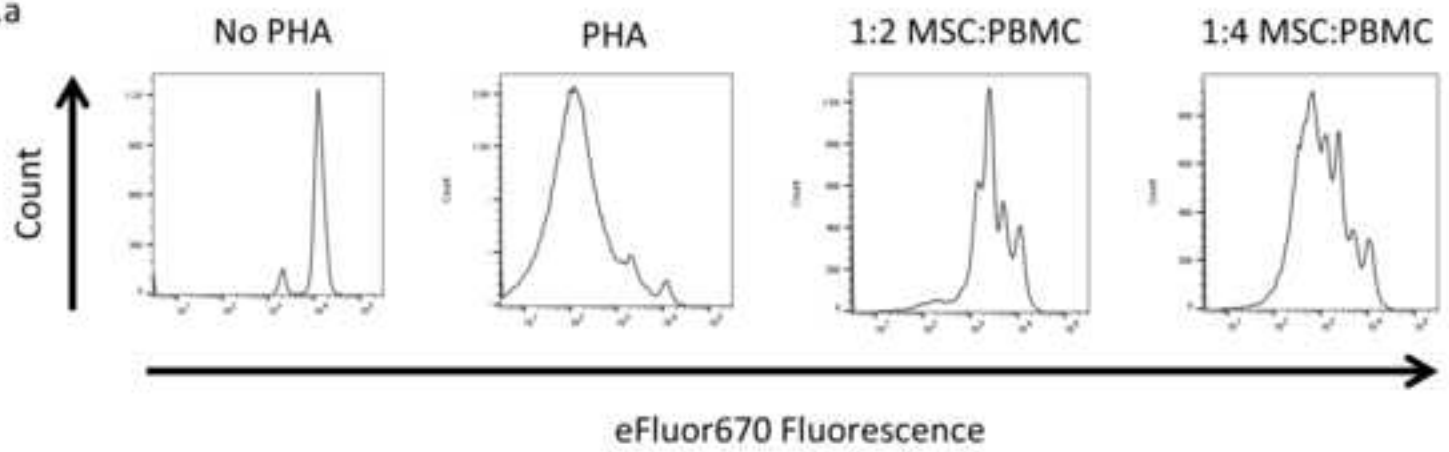


Fig.6.b

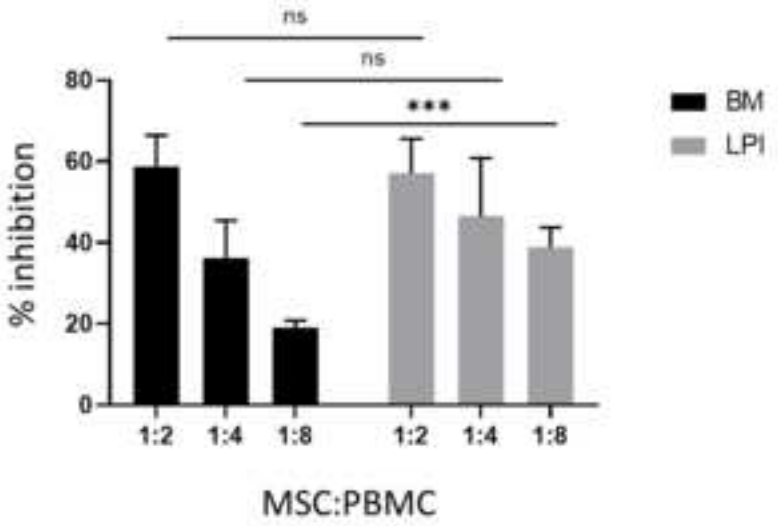


Fig.6.c

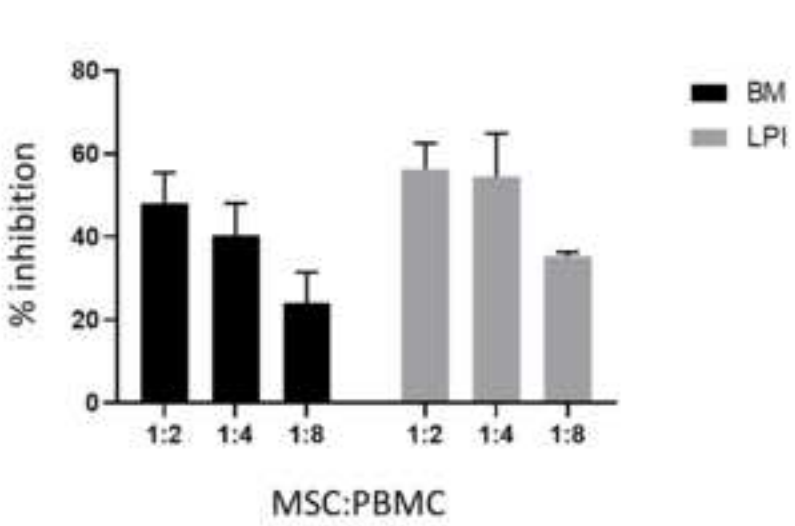


Figure 7

Fig.7.a

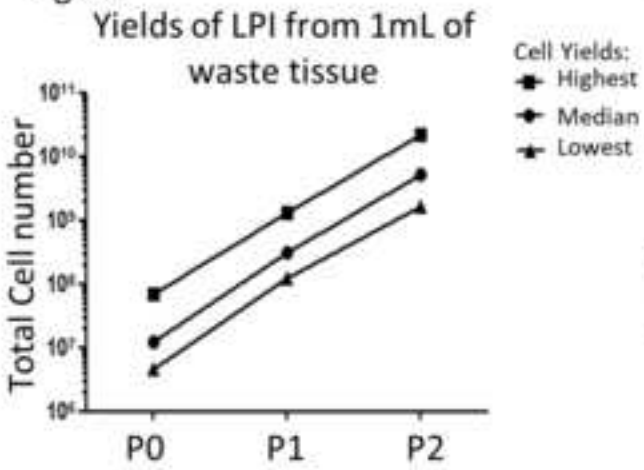


Fig.7.b

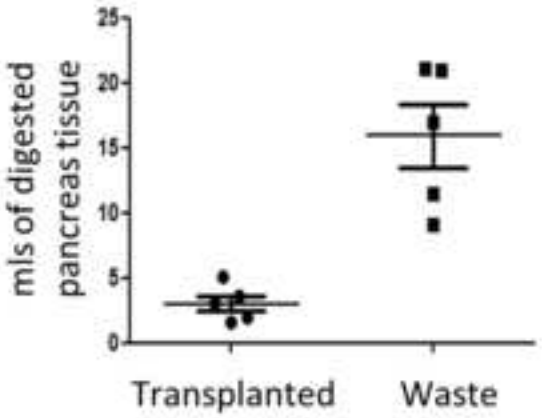


Fig.7.c

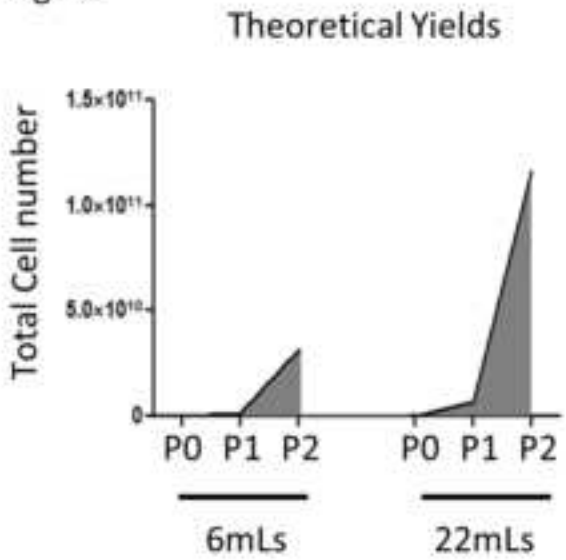


Fig.7.d